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United States Patent Application

Title of the Invention

METHODS AND APPARATUS FOR THE RECOVERY
OF NUCLEIC ACIDS

Inventors

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TITLE OF THE INVENTION

METHODS AND APPARATUS FOR THE RECOVERY OF NUCLEIC ACIDS

BACKGROUND OF THE INVENTION

1. Field of the invention

The present invention relates to a method for the recovery of nucleic acids from a nucleic acid-containing substance. In more detail, it relates to a method for the recovery of nucleic acids, which is suitable for an automatic device for the recovery of nucleic acid components as endogenous or exogenous genes from humor components for gene diagnosis by means of nucleic acid assays, or suitable for an automatic device for the recovery of plasmid DNAs from recombinant E. coli or the like for base sequencing of nucleic acids.

2. Description of the Related Art

A multitude of genetic technologies have been developed based upon advances in molecular biology, and a number of morbid genes have been isolated and identified according to these technologies. As a result, molecular biological techniques have been adopted ^{as} to techniques for diagnosis or examination in the field of medical care so as to enable diagnosis which have been unable to conduct or to shorten the period of examination remarkably.

The significant advances largely owe to gene amplification technique^s, ~~in~~^{and} particular to polymerase chain reaction (hereinafter referred to as PCR: Saiki et al., Science, 239, 487-491(1988)) technique^s.

The PCR technique enables nucleic acids in a solution to amplify sequence-specifically so that it provides, for example, an indirect proof of the presence of a trace quantity of a virus in serum by amplifying and detecting nucleic acids as the viral gene.

The PCR technique is, however, somewhat disadvantageous in the use for clinical daily examinations. In particular, there are some difficulties in extraction and purification steps of nucleic acids in a pretreatment according to this technique, whereas the extraction and purification steps of nucleic acids have been indicated to be key steps (Ooshima et al., JJCLA 22(2),145-150(1997)).

These difficulties are attributed to inhibitory factors remained ^{ing} in the purification step of nucleic acids, and known inhibitory factors include hemoglobin in blood, surfactant used in the extraction step and the like.

In addition, the extraction step requires a complicated procedure and a large amount of skilled labor. Therefore, this step is an obstacle to a new introduction of the genetic test into laboratories of hospitals, and the automatization of this step has been demanded.

On the contrary, plasmid DNAs are frequently used as materials for genetic engineering, and automatization of the extraction and purification steps of nucleic acids has been demanded from the viewpoint of labor savings in institutions for molecular biological researches, as well as in the laboratories.

SUMMARY OF THE INVENTION

As a method for the recovery of nucleic acids from a biological sample at a high purity and being free from inhibitory factors, there has been known a method for the recovery of nucleic acids comprising the steps of allowing a surfactant to act on a biological sample in the presence of a protease to release nucleic acids, mixing the released nucleic acids with phenol (and chloroform), repeating aqueous phase (water phase)-organic phase isolation by a centrifuge several times and then recovering the nucleic acids as sediments from the aqueous phase with the use of an alcohol.

This method, however, has some disadvantages due to the use of an organic solvent such as phenol, a toxic substance, in the steps. To be more specific, an organic solvent such as phenol has the possibilities of dissolving plastics of a device used for the recovery of nucleic acids and of deteriorating the device.

In addition, the use of an organic solvent such as phenol requires complicated treatments for discarding the organic solvent.

As other method for the recovery of nucleic acids than the method utilizing the aqueous phase-organic phase isolation, there have been reported a method for the recovery of DNAs from agarose gel utilizing the bonding properties of nucleic acids to the surface of glass in the presence of a chaotropic agent (B. Vogelstein and D. Gillespie, Proc. Natl. Acad. Sci. USA, 76(2), 615-619(1979)),

or a method for recovering plasmid DNAs from *E. coli* (M. A. Marko, R. Chipperfield and H. C. Birnboim, Anal. Biochem, 121,382-387(1982)).

B A method for the recovery of nucleic acids from a biological sample by means of a simpler procedure is described in Japanese Unexamined Patent Publication No. 2-289596. According to this method, nucleic acids can^{be} rapidly recovered by mixing a biological sample with a sufficient amount of a chaotropic agent (such as guanidinium salt~~s~~) and silica beads and binding the free nucleic acids to a solid phase.

B To obtain purified nucleic acids, however, this method requires a procedure for removing the guanidinium salt from the solid phase while ~~re~~maining the nucleic acids bound to the solid phase, whereas a suitable removing procedure is not described.

B For^{an} efficient washing procedure of a solid phase at room temperature, the use of ethanol at least in a 75% concentration has been recommended in the past (C. W. Chen and C. A. Thomas, Jr., Anal. Biochem., 101,339-342(1980)). As ethanol is a volatile component, the mechanization of the method requires a close-open mechanism of a lid or a cooling mechanism for preventing ethanol from evaporation, and this invites upsiz^{ing}~~ation~~ of the device or deterioration of its reliability.

B In addition, the method requires a washing step with ~~a~~ 70% ethanol and/or acetone and hence requires a removing procedure of acetone by drying. When ethanol at a

concentration of 70% or more and/or acetone, both of which are volatile, is mounted on an automatic device, the device requires a highly airtight chamber and a close-open mechanism of a lid and/or a cooling mechanism for preventing volatilization of the reagents, as described above.

The use of acetone has possibilities of dissolving plastics of a device employed for the recovery of nucleic acids and deteriorating the device due to its strong properties as an organic solvent, as mentioned above.

Therefore, materials of the device, cases and dispensers or other utensils to be used are remarkably limited. In addition, as acetone has acute toxicity and is inflammable, the release of acetone into the environment accompanied with the evaporation procedure should be avoided completely.

Japanese Unexamined Patent Publication No. 63-154696 discloses a method of isolating and purifying nucleic acids from an aqueous solution of a biological sample containing nucleic acids.

According to this method, an objective nucleic acid is recovered in the following manner: Initially, a biological sample is charged to a column filled with an anion exchanger, and the column is washed with an aqueous solution of sodium chloride to avoid the elution of an objective nucleic acid and to cleanse nonbinding components inclusive of carboxylated mucopolysaccharides. The objective nucleic acid is then recovered by eluting from the column with an aqueous solution of sodium chloride at such a concentration

as to elute the objective nucleic acid.

The method just mentioned above ensures the recovery of an objective nucleic acid without using harmful substances.

This method where a sodium chloride aqueous solution is used as a washing solution, however, provides only a low recovery of nucleic acids. Accordingly, demands have been made to provide a method for recovering nucleic acids at a higher recovery^{yield}/.
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It is, therefore, an object of the present invention to provide a method and apparatus for the recovery of nucleic acids from a material containing nucleic acids, by which nucleic acids can be recovered rapidly and easily at a high recovery^{yield}/and high purity without deteriorating the yield.
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It is another object of the invention to provide a method for the recovery of nucleic acids, which is suitable for apparatus for the automatic recovery of nucleic acids from a biological sample at a high recovery^{yield}/.
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A further object of the invention is to provide a method for the recovery of nucleic acids, which is suitable for apparatus for the automatic recovery of plasmid DNAs from *E. coli* at a high recovery^{yield}/.
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To achieve the above objects, the present invention provides the following constructions.

(1) The invention provides, in a first aspect, a method for the recovery of nucleic acids comprising a step 2 for mixing an accelerator substance for the binding of nucleic acids to a solid phase with a nucleic acid-containing material, a step 3 for making the mixture obtained in ~~the~~
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step 2 in contact with a solid phase bondable to nucleic acids to form a nucleic acid-binding solid phase, a step 4 for isolating the nucleic acid-binding solid phase from a liquid, a step 5 for washing the nucleic acid-binding solid phase with a solution containing an acetate, and a step 6 for eluting the nucleic acids from the solid phase.

(2) The method may preferably include a step 1 for promoting the release of nucleic acids from the nucleic acid-containing material before ~~the~~ step 2 in the above aspect.

(3) ~~The~~ ^S step 5 in the above method may preferably be composed of a step 5a for removing non-nucleic-acid components from the nucleic acid-binding solid phase by washing, and a step 5b for removing the accelerator substance from the solid phase by washing.

(4) The substance for removing non-nucleic-acid components from the solid phase in ~~the~~ step 5a may advantageously be guanidine hydrochloride.

(5) The acetate solution in the ¹st aspect may preferably be a solution containing 0.5 mol/liter or more of potassium acetate.

(6) In a second aspect the invention provides a method for the recovery of nucleic acids from a material containing nucleic acids, which includes a step 2 for mixing an accelerator substance for the binding of nucleic acids to a solid phase with a nucleic acid-containing material, a step 3 for making the mixture obtained in the step 2 in contact with a solid phase bondable to nucleic acids to form a

B nucleic acid-binding solid phase, a step 4 for isolating the nucleic acid-binding solid phase from a liquid, a step 5 for washing the nucleic acid-binding solid phase with a solution containing 0.2 mole/liter or more of potassium chloride, and a step 6 for eluting the nucleic acids from the solid phase.

(7) The invention in a third aspect provides a method for the recovery of nucleic acids from a material containing nucleic acids, which is composed of a step 2 for mixing an accelerator substance for the binding of nucleic acids to a solid phase with a nucleic acid-containing material, a step 3 for making the mixture obtained in the step 2 in contact with a solid phase bondable to nucleic acids to form a nucleic acid-binding solid phase, a step 4 for isolating the nucleic acid-binding solid phase from a liquid, a step 5 for washing the nucleic acid-binding solid phase with a mixture of an aqueous solution of a salt and an alcohol, and a step 6 for eluting the nucleic acids from the solid phase.

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B (8) The method described in the third aspect may preferably have a step 7 for removing the alcohol after the elution of nucleic acids from the solid phase in ~~the~~ step 6.

B (9) The alcohol used in ~~the~~ step 5 described in the third aspect may preferably be ethanol at a concentration of less than 50% in the mixture.

B (10) The washing solution in ~~the~~ step 5 described in the third aspect may advantageously be a solution containing 40% of ethanol and 10 mmol/liter or more of potassium acetate.

B (11) The washing solution used in ~~the~~ step 5 described

in the third aspect may be a solution containing 40% of ethanol and 25 mmol/liter or more of sodium chloride.

3 (12) The accelerator substance used in ~~the~~ step 2 in the above aspects may preferably be guanidine hydrochloride.

(13) Preferably, the solid phase bondable to nucleic acids may be a substance containing silicon dioxide.

(14) In a further aspect, the invention provides apparatus for the recovery of nucleic acids from a material containing nucleic acids, which is composed of a first means for mixing nucleic acids with an accelerator substance for the binding of nucleic acids to a solid phase, a second means for making the mixture in contact with a solid phase bondable to nucleic acids to form a nucleic acid-binding solid phase, a third means for isolating the nucleic acid-binding solid phase from a liquid, a fourth means for washing the nucleic acid-binding solid phase with a solution containing an acetate, and a fifth means for eluting the nucleic acids from the solid phase.

(15) The invention provides in yet another aspect apparatus for the recovery of nucleic acids from a material containing nucleic acids, which is composed of: a first pipetter for discharging an accelerator substance for the binding of nucleic acids to a solid phase, a solid phase bondable to nucleic acids, a washing solution for removing the accelerator substance from the solid phase, and a liquid for eluting nucleic acids from the solid phase, separately and in turn, into a chamber encasing a nucleic acid-containing material and for stirring the liquid mixture in

the chamber, an isolating means for isolating the solid phase from the liquid phase in the chamber, a second pipetter for aspirating the liquid phase isolated by the isolating means from the chamber, and a control means for controlling operations of the first pipetter, the isolating means and the second pipetter, wherein the liquid which has been eluted from the solid phase by an eluent for eluting nucleic acids and isolated by the isolating means as a liquid phase is aspirated by the second pipetter, and an aqueous solution containing the nucleic acids is purified from the aspirated liquid.

When guanidine hydrochloride (hereinafter briefly referred to as GuHCl) is used as the accelerator substance for the binding of nucleic acids to a solid phase in ~~the~~ step 1 of the above methods, such effects on absorbance at 260 nm as to adversely affect the assay of the purity or quantity of nucleic acids can be markedly reduced.

As the solid phase bondable to nucleic acids in ~~the~~ step 2, any substance containing silicon oxide such as glass beads, silica powder, quartz³ filter paper, quartz² wool or crushed products of these substances, diatomaceous earth and the like can be employed. When the solid phase has a particle size ranging from about 1 to about 100 μ m, a practically sufficient binding of nucleic acids to the solid phase can be obtained in several minutes to ten minutes.

The use of diatomaceous earth can reduce a cost necessary for one treatment. When a stirring procedure is also employed for enhancing the contact probability of

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These and other features, objects and advantages of the present invention will become apparent upon a consideration of the following detailed description of the invention when read in conjunction with the drawings, in which:

Fig. 1 is an operating flow chart for the recovery of nucleic acids according to a first embodiment of the invention;

Fig. 2 is an operating flow chart for the recovery of nucleic acids inclusive of a washing step with a solution containing potassium acetate or the like according to a second embodiment of the invention;

Fig. 3 is an operating flow chart for the recovery of nucleic acids inclusive of a washing step according to third, fourth, fifth and sixth embodiments of the invention;

Fig. 4 is an operating flow chart inclusive of a washing step only with an aqueous ethanol solution according to conventional techniques for the recovery of nucleic acids.

Fig. 5 is a graphical diagram illustrating the results of the washing step with ethanol according to conventional techniques;

Fig. 6 is a graphical diagram illustrating the results of the washing step according to the second embodiment of the invention;

Fig. 7 is a graphical diagram showing the results of the washing step according to the third embodiment invention;

Fig. 8 is a graphical diagram indicating the results of

the washing step according to the fourth embodiment of the invention;

Fig. 9 is a plan view of a layout of apparatus for the recovery of nucleic acids as a seventh embodiment of the invention;

Fig. 10 is a schematic block diagram illustrating apparatus for the recovery of nucleic acids; and

Fig. 11 is a simplified outside drawing of apparatus for the recovery of nucleic acids.

10 DESCRIPTION OF THE PREFERRED EMBODIMENT

11 Embodiment 1

Recovery of nucleic acids using an aqueous solution of an acetate at a concentration of 1 mol/liter or the like in the washing step

Nucleic acids were recovered from an aqueous solution containing nucleic acids according to the flow chart in Fig. 1 in the following manner. A nucleic acid-containing material obtained by diluting a commercially available purified pBR322 DNA with a Tris-EDTA (hereinafter referred to as TE) buffer (pH of 7.4) was used.

β In this case, nucleic acids were already free in the material, and ~~the~~ step 1 for promoting the release of nucleic acids from the material was omitted. As the accelerator substance generally used in ~~the~~ step 1, there may be mentioned surfactant, enzymes and others. The means for promoting the release of nucleic acids from the nucleic acid-containing material includes a heating means and a

B means of applying an ultrasonic^{energy} for example.

In the step 2 for mixing the released nucleic acids with an accelerator substance for the binding of nucleic acids to a solid phase, 900 μ l of a 6 mol/liter GuHCl solution was added to 100 μ l of the above aqueous solution containing nucleic acids and stirred in a 1.5-ml reactor.

B ~~The~~ ^S step 3 for making the mixture in contact with a solid phase bondable to nucleic acids was carried out by adding 100 mg of glass beads as the solid phase to the mixture and stirring the resultant mixture at room temperature by a rotary mixer to make the solid phase in contact with a liquid to form a nucleic acid-binding solid phase.

B In the ~~fourth~~ ⁴ step for isolating the nucleic acid-binding solid phase from a liquid, the mixture was centrifuged at 15,000 g for 1 minute with a centrifuge to give a sediment of the solid phase and the supernatant was discarded.

B ~~The~~ ^S step 5 for washing the nucleic acid-binding solid phase with a solution containing an acetate or the like was conducted by suspending the solid phase in ~~an~~ 1 mol/liter aqueous solution of an acetate or other reagents indicated in Table 1, and discarding the supernatant.

B In ~~the~~ step 6 for eluting nucleic acids from the solid phase, a TE buffer (pH 8.0) was added to the solid phase and heated at 60°C for 1 minute for elution, and the elute was centrifuged at 15,000 g for 1 minute to give a supernatant. A part of the supernatant was subjected as a sample to electrophoresis using a 0.8% agarose gel as a support and

subsequently to ethiodium bromide staining, and the intensities of individual bands were converted into numbers by densitography so as to calculate the recovery based on the amount of nucleic acids in ~~the~~ step 1.

The results are shown in Table 1. These results demonstrate that the use of ammonium chloride or potassium acetate provides efficient maintenance of nucleic acids in the washing as compared to the use of sodium chloride.

Table 1

Salt	Recovery
Lithium chloride	1.8%
Sodium chloride	76.3%
Potassium chloride	43.0%
Ammonium chloride	81.0%
Sodium acetate	12.5%
Potassium acetate	81.9%
Ammonium acetate	5.2%
Tris buffer	0.9%

As described above, the method according to Embodiment 1 of the invention includes a step 1 for promoting the release of nucleic acids from a nucleic acid-bearing material, a step 2 for mixing the released nucleic acids with an accelerator substance for the binding of nucleic acids to a solid phase, a step 3 for making the mixture in contact with a solid phase bondable to nucleic acids to form a nucleic acid-binding solid phase, a step 4 for isolating the solid phase from a liquid, a step 5 for washing the nucleic acid-binding solid phase with a solution containing ammonium chloride or potassium acetate, and a step 6 for eluting nucleic acids from the solid phase.

Embodiment 1 of the invention provides a method for the recovery of nucleic acids which can rapidly and easily recover high-purity nucleic acids from a material containing nucleic acids at a high recovery^{yield} without reducing the yield. In other words, nucleic acids at a suitable purity for

genetic tests or gene analyses can be rapidly and easily recovered without the use of hazardous substances.

In addition, this embodiment provides a method for the recovery of nucleic acids, which is suitable for apparatus for automatic recovery of nucleic acid components in a biological sample, because it does not require the use of volatile substances or substances deteriorating the apparatus.

Accordingly, it provides a method for the recovery of nucleic acids, which is desirably applicable to apparatus for the automatic recovery of plasmid DNAs from *E coli*.

Embodiment 2

Recovery of nucleic acids using a solution containing potassium chloride or potassium acetate in the washing step

Nucleic acids were recovered from an aqueous solution containing nucleic acids in accordance with the flow chart shown in Fig. 2. ~~The~~ ⁵ steps 1, 2, 3, 4 and 6 in the flow chart in Fig. 2 are similar to those in the flow chart of Fig. 1, whereas ~~the~~ step 5 in Fig. 1 is divided into ~~the~~ steps 5a and 5b in Fig. 2.

A material containing nucleic acids obtained by diluting a commercially available purified pBR322 DNA with a TE buffer (pH 7.4) was employed in Embodiment 2, as similar to Example 1.

The description of ~~the~~ steps 1 to 4 is omitted because these steps are the same as those of Fig. 1.

~~The~~ ⁵ step 5a is a step for removing non-nucleic-acid components from the solid phase by washing the nucleic acid-

binding solid phase with a solution containing a salt. The solid phase was washed by suspending the solid phase in 1,000 μ l of a 6 mol/liter guanidine hydrochloride (GuHCl) solution, centrifuging the suspension at 15,000 g for 1 minute to obtain a sediment of the solid phase and discarding the supernatant.

In ~~the~~ step 5b for washing the nucleic acid-binding solid phase with a solution containing a salt to remove the accelerator substance from the solid phase, the solid phase was suspended respectively in aqueous solutions of NaCl, KCl, NaOAc (sodium acetate) or KOAc (potassium acetate) having different concentrations and the supernatant was discarded.

~~The~~ ⁵ step 6 was conducted as follows: A TE buffer (pH 8.0) was added to the solid phase, the mixture was heated at 60°C for 1 minute for elution, the elute was centrifuged at 15,000 g for 1 minute to obtain a supernatant, a part of the supernatant as a sample was subjected to electrophoresis with the use of a 0.8% agarose gel as a support, then to ethidium bromide staining, the intensities of individual bands were converted into numbers by densitography, and the recovery was calculated based upon the amounts of nucleic acids in ~~the~~ step 1.

The results are set forth in Fig. 6. When NaCl or KOAc was used at a concentration of 500 mmol/liter or more, a recovery of equal to or more than 50% was obtained, whereas when KCl was used at a concentration of 200 mmol/liter or more, a recovery of equal to or more than 50% was obtained.

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These results demonstrate that the use of KCl or KOAc provides, at least, more efficient recovery of nucleic acids than the use of NaCl^{at} at least a concentration of 800 mmol/liter or less.

As described above, Embodiment 2 according to the invention where KCl or KOAc is used for washing non-nucleic acid components or the like can obtain similar advantages to Embodiment 1.

Embodiment 3

Recovery of nucleic acids using a solution containing sodium acetate in the washing step

Nucleic acids were recovered from an aqueous solution containing nucleic acids in accordance with the flow chart shown in Fig. 3. ~~The~~ ^S steps 1, 2, 3, 4, 5a and 6 in the flow chart of Fig. 3 are the same as in the flow chart of Fig. 2, hence the description of ~~the~~ steps 1 to 5a is omitted.

~~The~~ ^S step 5b was conducted by suspending the solid phase in aqueous solutions of 40% ethanol and KOAc or NaCl having different concentrations, and discarding the supernatant.

~~The~~ ^S step 6 was carried out by adding a TE buffer (pH 8.0) to the solid phase, heating the mixture at 60°C for 1 minute for elution, centrifuging the elute at 15,000 g for 1 minute to give a supernatant. In the step 7 for removing alcohol, ethanol was removed by heating at 60°C to give an aqueous solution of purified nucleic acids.

A part of the solution was subjected as a sample to electrophoresis using a 0.8% agarose gel, and then to ethidium bromide staining, the intensity of each band was

converted into numbers by densitography, and the recovery was calculated based upon the amounts of nucleic acids in ~~the~~ step 1.

The results are indicated in Fig. 7. When used in combination with 40% ethanol, the use of KOAc can more efficiently recover nucleic acids than the use of NaCl at a concentration ranging from 10 mmol/liter to 25 mmol/liter.

According to Embodiment 3 of the invention, similar advantages to Embodiment 1 can be obtained with the use of KOAc at a concentration ranging from 10 mmol/liter to 25 mmol/liter.

Embodiment 4

Recovery of nucleic acids with the use of a combination of ethanol with solutions of sodium acetate or sodium chloride at different ethanol concentrations in the washing step

In accordance with the flow chart indicated in Fig. 3, nucleic acids were recovered from an aqueous solution containing nucleic acids. ~~The~~ ^S steps 1, 2, 3, 4, 5a and 6 in the flow chart of Fig. 3 are the same as in the flow chart of Fig. 2, hence the description of ~~the~~ steps 1 to 5a is omitted.

~~The~~ ^S step 5b is a step for removing the accelerator substance from the solid phase by washing the nucleic acid-binding solid phase with a solution containing a salt. ~~The~~ ^S step 5b was conducted by suspending the solid phase in 25 mmol/liter or 50 mmol/liter of KOAc or 50 mmol/liter or 100 mmol/liter of NaCl in combination with different

concentrations of ethanol, and discarding the supernatant.

~~The~~ ⁵Step 6 was carried out by adding a TE buffer (pH 8.0) to the solid phase, heating the mixture at 60°C for 1 minute for elution, centrifuging the elute at 15,000 g for 1 minute to give a supernatant. In ~~the~~ step 7 for removing alcohol, ethanol was removed by heating the mixture at 60°C to give an aqueous solution of purified nucleic acids.

A part of the solution was subjected as a sample to electrophoresis using a 0.8% agarose gel, and then to ethidium bromide staining, the intensity of each band was converted into numbers by densitography, and the recovery was calculated based upon the amounts of nucleic acids in ~~the~~ step 1.

The results are shown in Fig. 8. The combination use of 50 mmol/liter or more of KOAc or 100 mmol/liter or more of NaCl with ethanol can effectively prevent the recovery^{rate} from falling when the concentration of ethanol varies.

As mentioned above, Embodiment 4 of the invention can provide similar advantages to Embodiment 1. Incidentally, the method according to Embodiment 4 does not invite the same disadvantages as conventional equivalents even though ethanol is employed, because ethanol is used in combination with 25 mmol/liter or 50 mmol/liter of KOAc or 50 mmol/liter or 100 mmol/liter of NaCl.

Embodiment 5

Recovery of nucleic acids added to serum

Nucleic acids added to serum were recovered in accordance with the flow chart shown in Fig. 3.

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A sample obtained by adding a commercially available purified pBR322 DNA to human serum was used. Nucleic acids in this sample were already free, but sodium dodecyl sulfate (SDS) was added to the sample in ~~the~~ step 1 as measures against nuclease.

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~~The~~ ^S steps 2 to 5a are the same as in Embodiment 4, and the description of these steps is omitted.

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In ~~the~~ step 5b, the solid phase was suspended in a 50% ethanol aqueous solution containing 50 mmol/liter of KOAc and the supernatant was discarded.

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~~The~~ ^S step 6 was carried out by adding a TE buffer (pH 8.0) to the solid phase, heating the mixture at 60°C for 1 minute for elution, ^{and} centrifuging the elute at 15,000 g for 1 minute to give a supernatant. In ~~the~~ step 7 for removing alcohol, ethanol was removed by heating at 60°C to give an aqueous solution of purified nucleic acids.

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A part of the solution was subjected as a sample to electrophoresis using a 0.8% agarose gel, and then to ethidium bromide staining, the intensity of each band was converted into numbers by densitography, and the recovery ^{rate} ~~was~~ calculated based upon the amounts of nucleic acids in ~~the~~ step 1. The recovery was 70% and A_{260}/A_{280} was 1.90. The total time required for ~~the~~ steps 1 to 7 was about 30 minutes.

The method according to Embodiment 5 can also provide similar advantages to Embodiment 1 mentioned above. Even though ethanol is used in Embodiment 5, disadvantages as in conventional equivalents do not occur because a 50% ethanol

aqueous solution containing 50 mmol/liter of KOAc is employed.

Embodiment 6

Recovery of plasmid DNAs from cultured recombinant *E. coli*

Plasmid DNAs were recovered from cultured recombinant *E. coli* in accordance with the flow chart of Fig. 3. As a sample, *E. coli* obtained by integrating pBR322 DNA into *E. coli* HB101 in a genetic engineering manner. The sample was obtained in the following manner: *E. coli* was cultured in 1 ml of LB medium at 37°C for one night and cells were collected by centrifugation, then suspended in 100 µl of a 0.15 mol/liter solution of NaCl to give a sample.

In ~~the~~ step 1, a solution containing 50 mmol/liter of glucose, 10 mmol/liter of EDTA and 25 mmol/liter of Tris-HCl buffer (pH 8.0) with 8 mg/ml of lysozyme was added to the sample for destroying peptidoglycan in the cell wall of *E. coli*.

Further, a solution of 0.2 mol/liter of NaOH and 1% of SDS was added to the resultant mixture for the release of nucleic acids. To this solution was added a 5 mol/liter potassium acetate solution to give an aqueous solution containing nucleic acids.

A total of 1,000 µl of a 6 mol/liter GuHCl solution was added to the above aqueous solution and stirred in ~~the~~ step 2.

~~The~~ ⁵ step 3 was conducted by adding 150 mg of glass beads as a solid phase bondable to nucleic acids to the

above mixture and stirring the resultant mixture at room temperature by a rotary mixer to make the solid phase in contact with a liquid and to form a nucleic acid-binding solid phase.

In ~~the~~ step 4 for isolating the nucleic acid-binding solid phase from a liquid, the mixture was subjected to centrifugation at 15,000 g for 1 minute to give a sediment of the solid phase, and the supernatant was discarded.

~~The~~ ^S step 5a was carried out for washing the solid phase by suspending the solid phase in 1,000 μ l of a 6 mol/liter GuHCl solution, centrifuging the suspension at 15,000 g for 1 minute to give a sediment of the solid phase, and discarding the supernatant.

In ~~the~~ step 5b, the solid phase was suspended in a 50% ethanol aqueous solution containing 50 mmol/liter of KOAc, and the supernatant was discarded.

~~The~~ ^S step 6 was carried out by adding a TE buffer (pH 8.0) to the solid phase, heating the mixture at 60°C for 1 minute for elution, centrifuging the elute at 15,000 g for 1 minute to give a supernatant. In ~~the~~ step 7 for removing alcohol, ethanol was removed by heating at 60°C to give an aqueous solution of purified nucleic acids. A part of the solution was subjected as a sample to electrophoresis using a 0.8% agarose gel, and then to ethidium bromide staining to give electrophoresis bands of nucleic acids. As a result, bands of plasmid DNA and rRNA were determined in comparison with a molecular weight marker which was subjected to electrophoresis simultaneously.

B
The A_{260}/A_{280} was 1.96 at this step. The yield of plasmid DNAs was 4.5 μ g as calculated from absorbencies of A_{260} after treatments with ribonuclease and ethanol precipitation. The total time required for ~~the~~ steps 1 to 7 was about 45 minutes.

The method according to Embodiment 6 can provide similar advantages to Embodiment 1 mentioned above. Even though ethanol is used in Embodiment 6, disadvantages as in conventional equivalents are not invited, because a 50% ethanol aqueous solution containing 50 mmol/liter of KOAc is employed.

Embodiment 7

Recovery of DNAs added to serum by an automatic device for the recovery of nucleic acids

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B6220T-88T6ZT60
Fig. 9 is a plan view of a layout of ^{an} apparatus (automatic device) for the recovery of nucleic acids as Embodiment 7 of the invention; Fig. 10 is a schematic block diagram illustrating the apparatus; and Fig. 11 is a simplified ~~outside~~ ^{outside of the} drawing of the apparatus.

In Fig. 10, a personal computer (PC) 124 as an operation control unit is connected to a keyboard 125 as a control panel for input of operation conditions of the apparatus or information on test samples, a CRT 123 as a display for showing input information, alarm information or others, and a mechanical control unit 126 for controlling each mechanical unit of the apparatus. The personal computer 124 includes a memory for holding data and programs.

The mechanical control unit 126 controls a stepping motor 127 for moving an agitator 101 circumferentially and vertically, and a stepping motor 128 for moving a pipetter A 102 circumferentially and vertically. The mechanical control unit 126 also controls a stepping motor 129 for circumferentially and vertically moving a pipetter B 103, a stepping motor 130 for horizontally moving a reactor transport mechanism 104, and a stepping motor 131 for horizontally moving a transport mechanism 105 for a purified product container.

The agitator 101 as shown in Figs. 9 to 11 is movable circumferentially and vertically, and movable between an inlet 107 for particles and a washing position 108 as a function of the mechanical control unit 126. The pipetter A 102 and the pipetter B 103 are movable circumferentially and vertically, and respectively have a nozzle 118 and a nozzle 119 for mounting a disposable tip.

The reactor transport mechanism 104 can transport a reactor 120 to point A, B or C as a function of the mechanical control unit 126, and a permanent magnet 122 ^{is} ~~was~~ placed on both sides of the point C in such a shape as to fit the size of the reactor 120. The transport mechanism 105 is capable of transporting a purified product container 121 to points D and E as a function of the mechanical control unit 126.

Nucleic acids added to serum were recovered by the apparatus illustrated in Fig. 9 in accordance with the flow chart of Fig. 3.

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A sample obtained by adding a commercially available purified pBR322 DNA to human serum was dispensed to the reactor 120 in a given volume, and the reactor was placed at the point A. Although the nucleic acids in this sample were already free, SDS was added to the sample in ~~the~~ step 1 for measures against nuclease.

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In ~~the~~ step 2, the reactor 120 was moved to the point B by the reactor transport mechanism 104, ^{and} the pipetter A 102 was moved to a tip mount position 106 by rotating circumferentially to mount a tip on the nozzle 118. Subsequently, the pipetter A 102 was moved to an inlet 109 to aspirate 900 μ l of a 8 mol/liter GuHCl solution relative to 100 μ l of the aqueous solution containing nucleic acids.

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The pipetter A 102 was then moved to the point B so as to discharge GuHCl into the reactor 120. The aspiration-discharging operation was then repeated several times for stirring. The pipetter A 102 was then moved to a tip-discarding position 116 so as to discard the tip from the nozzle 118.

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In ~~the~~ step 3, magnetic particles in a liquid was used as a solid phase bondable to nucleic acids, and the liquid was stirred by the agitator 101 from the inlet 107, whereas a new tip was mounted to the nozzle 118 of the pipetter A 102 at the tip mount position 106. The stirred liquid containing magnetic particles was aspirated through the new tip. The pipetter A 102 was then moved to the point B, and the content of the tip was discharged into the reactor 120. The aspiration-discharging procedure was repeated for

stirring. The agitator 101 was moved to the washing position 108 and washed, whereas the pipetter A 102 was moved to the tip discarding position 116 to discard the tip from the nozzle 118.

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~~The~~ step 4 was conducted for isolating the nucleic acid-bearing solid phase from a liquid in the following manner: Initially, the reactor 120 was moved from the point B to the point C by the reactor transport mechanism 104 and magnetic particles in the reactor 120 were ^{attached onto} ~~grappled on~~ the side wall of the reactor by means of the permanent magnet 122. Next, the pipetter B 103 was moved to a tip mount position 113 by rotation to mount a tip on the nozzle 119, and then moved to the point C so as to aspirate a liquid phase in the reactor 120. The pipetter B was then moved to a solution-discarding position 114 so as to discard the solution from the tip, and moved to a tip-discarding position 115 to discard the tip from the nozzle 119.

In ~~the~~ step 5a, the reactor 120 was moved from the point C to the point B by means of the reactor transport mechanism 104, and the pipetter A 102 was moved to the tip mount position 106 to mount a tip on the nozzle 118. Subsequently, the pipetter A 102 was moved to an inlet 111 to aspirate the GuHCl solution at a given volume, and then moved to the point B to discharge the GuHCl solution into the reactor 120. After stirring the mixture by the aspiration-discharging operation, the pipetter was moved to the tip-discarding position 116 to discard the tip from the nozzle 118.

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Subsequently, the reactor 120 was moved from the point B to the point C by means of the reactor transport mechanism 104, and the magnetic particles in the reactor 120 were ~~grappled on~~ ^{attached onto} the side wall of the reactor by the permanent magnet 122. The pipetter B 103 was then moved to the tip mount position 113 by rotation to mount a tip on the nozzle 119, and then moved to the point C to aspirate a liquid phase in the reactor 120. The pipetter B was then moved to the solution-discarding position 114 so as to discard the solution from the tip, and moved to the tip-discarding position 115 to discard the tip from the nozzle 119.

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In ~~the~~ step 5b, the reactor 120 was moved from the point C to the point B by the reactor transport mechanism 104. Next, the pipetter A 102 was moved to the tip mount position 106 to mount a tip to the nozzle 118, and moved to an inlet 110 to aspirate a given volume of a 50% ethanol aqueous solution containing 50 mmol/liter of KOAc. Subsequently, the pipetter was moved to the point B to discharge the ethanol solution into the reactor 120. After stirring the content of the reactor by the aspiration-discharging operation, the pipetter was moved to the tip-discarding position 116 to discard the tip from the nozzle 118.

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Further, the reactor 120 was moved from the point B to the point C by the reactor transport mechanism 104 and magnetic particles in the reactor 120 were ~~grappled on~~ ^{attached onto} the side wall of the reactor by means of the permanent magnet 122. Next, the pipetter B 103 was moved to the tip mount

position 113 by rotation to mount a tip on the nozzle 119, and then moved to the point C so as to aspirate a liquid phase in the reactor 120. The pipetter was then moved to the solution-discarding position 114 so as to discard the solution in the tip, and moved to the tip-discarding position 115 to discard the tip from the nozzle 119. This step was further repeated a given number of times.

B In ~~the~~ step 6, the reactor 120 was moved from the point C to the point B by the reactor transport mechanism 104, and the pipetter A 102 was moved to the tip mount position 106 to mount a tip on the nozzle 118, then moved to the inlet 109 to aspirate a given volume of a TE buffer (pH 8.0) heated at 60°C, and moved to the point B. The heated TE buffer was then discharged into the reactor 120 to stir the contents by the aspiration-discharging operation, and the pipetter A was moved to the tip-discarding position 116 to discard the tip from the nozzle 118.

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Subsequently, the reactor 120 was moved from the point B to the point C by the reactor transport mechanism 104, and the magnetic particles in the reactor 120 were ~~grappled on~~ ^{attached onto} the side wall of the reactor by the permanent magnet 122, and the pipetter B 103 was then moved to the tip mount position 113 by rotation to mount a tip on the nozzle 119. The pipetter was then moved to the point C to aspirate a liquid phase in the reactor 120 and moved to the point D to discharge the solution into the purified product container 121. This step was further repeated a given number of times.

B In ~~the~~ step 7, the purified product container 121 was moved to the position E on a heat block 112 at 60°C by the transport mechanism 105, and heated for a given period to obtain an aqueous solution containing purified nucleic acids.

B A part of the obtained solution was subjected as a sample to electrophoresis using a 0.8% agarose gel as a support, then to ethidium bromide staining. The intensities of individual bands were converted into numbers by densitography, and the recovery was calculated based upon the amounts of nucleic acids in ~~the~~ step 1. The resultant recovery was 65%. The total time required for ~~the~~ steps 1 to 7 was about 30 minutes.

B As thus described, ^{an} apparatus for the recovery of nucleic acids can be obtained according to Embodiment 7 of the invention, by which nucleic acids can ^{be} rapidly and easily recovered at a high purity without deteriorating the yield. Even though ethanol is used in Embodiment 7, disadvantages as in conventional equivalents are not invited, because a 50% ethanol aqueous solution containing 50 mmol/liter of KOAc is employed.

B The operation of the apparatus as described above is carried out by a program ^e held in the memory of the personal computer 124. The program can also be ^{executed} ~~conducted~~ by recording ^{it} in a recording medium such as a compact disc (CD) separately and reading from the recording medium. The recording medium can surely be taken out from the apparatus for recovery.

Next, a comparative example will be described for the comparison between the present invention and related art.

Comparative Example

Recovery of nucleic acids using only an aqueous ethanol solution in the washing step as in the case of conventional equivalents

Nucleic acids were recovered from an aqueous solution containing nucleic acids in accordance with the flow chart of Fig. 4. A nucleic acid-bearing material obtained by diluting a commercially available pBR322 DNA with a TE buffer (pH 7.4) was used.

~~The~~ ^SStep 1 for promoting the release of nucleic acids from a nucleic acid-bearing material was omitted, since the nucleic acids in the material were already free. ~~The~~ ^SStep 2 for mixing the free nucleic acids with an accelerator substance for binding of nucleic acids to a solid phase was conducted by adding 900 μ l of a 6 mol/liter GuHCl solution to 100 μ l of the aqueous solution of nucleic acids and stirring the mixture in a 1.5-ml reactor.

In ~~the~~ step 3 for making the mixture in contact with a solid phase bondable to nucleic acids, 100 mg of glass beads as the solid phase was added to the mixture, and the resultant mixture was stirred at room temperature by a rotary mixer so as to make the solid phase in contact with a liquid and to form a nucleic acid-binding solid phase.

In ~~the~~ step 4 for isolating the nucleic acid-binding solid phase from a liquid, the mixture was centrifuged at 15,000 g for 1 minute using a centrifuge to give a sediment

of the solid phase, and the supernatant was discarded.

~~The~~ ^S step 5 for washing the nucleic acid-binding solid phase with an aqueous ethanol solution was carried out by suspending the solid phase respectively in aqueous ethanol solutions having different concentrations, centrifuging the suspension at 15,000 g for 1 minute to give a sediment of the solid phase, and discarding the supernatant.

~~The~~ ^S step 6 was conducted as follows: A TE buffer (pH 8.0) was added to the solid phase, the mixture was heated at 60°C for 1 minute for elution, the elute was centrifuged at 15,000 g for 1 minute to obtain the supernatant, a part of the supernatant was subjected as a sample to electrophoresis with the use of a 0.8% agarose gel as a support, then to ethidium bromide staining, the intensities of individual bands were converted into numbers by densitography, and the recovery was calculated based upon the amounts of nucleic acids in ~~the~~ step 1.

The results are indicated in Fig. 5. It was confirmed that the recovery of nucleic acids decreases with its peak at an ethanol concentration of about 80%, that is, the recovery is maximized at an ethanol concentration of 80%. In such a method, however, the use of ethanol at a high concentration is required and ethanol is volatile as described above. Accordingly, when such an ethanol solution is mounted on an automatic device, the device requires a highly airtight chamber and a close-open mechanism of a lid and/or a cooling mechanism for preventing volatilization of ethanol, and this complicates the construction of ^{the} device.

On the contrary, the aforementioned Embodiments 1 to 7 according to the invention can provide methods and apparatus for the recovery of nucleic acids, by which nucleic acids having a high purity can be rapidly and easily recovered from a material containing nucleic acids without deteriorating the yield.

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The present invention can be applied to any material containing nucleic acids, and the preferred materials include, for example, whole blood, serum, expectoration, urine and other clinical samples, cultured cells, cultured bacteria and other biological samples, and nucleic acids retained on a gel after electrophoresis.

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The methods according to the invention are also effective for materials containing DNA amplified enzymes and other reaction products or crude nucleic acids. The term "nucleic acid" used herein means and includes double-stranded, single-stranded, partially double-stranded or partially single-stranded DNAs or RNAs.

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The process for promoting the release of nucleic acids from a nucleic acid-bearing material in ~~the~~ step 1 according to the invention includes, mechanical processes using mortars, ultrasound or microwaves or the like, chemical processes using surfactant, denaturants or the like, biochemical processes ~~using, for instance,~~ and combinations of these processes.

As examples of the accelerator substance for the binding of nucleic acids to a solid phase, there may be mentioned NaI (sodium iodide), KI (potassium iodide), NaClO_4 ,

NaSCN (sodium thiocyanate), GuSCN (guanidine thiocyanate) and other chaotropic agents. These substances, however, have a lower end of the peak absorbance at around 260 nm, at which nucleic acids have the peaks. Therefore, if any of these substances contaminates the purified nucleic acid solution, it can adversely affect the assay results of purity or quantity of the nucleic acids with the use of a spectrophotometer, whereas such a spectrophotometer is generally employed in the assays.

Further, NaSCN and GuSCN each containing thiocyanic acid produce lethal HCN (hydrogen cyanide) by a reaction with an acid, waste containing these substances should be treated with care.

Accordingly, GuHCl (guanidine hydrochloride) should preferably be used as the accelerator substance in the present invention. GuHCl is easily available and invites a remarkably lower adverse effect on the absorbance at 260 nm than the other chaotropic agents. In addition, it has no potential risk of producing a lethal gas.

The final concentration of GuHCl in the invention should advantageously fall in the range from 4 to 6 mol/liter for the specific binding of nucleic acids ~~component~~ to a solid phase.

As the solid phase bondable to nucleic acids used in ~~the~~ step 3 according to the invention, any silicon dioxide-containing substance can be employed, including glass beads, silica powder, quartz filter paper, quartz wool or crushed products of these substances, and diatomaceous earth.

Preferably, the use of particles having a small particle size is advantageous for enhancing the contact probability between nucleic acids and the solid phase in the mixture of nucleic acids and a chaotropic agent, and hence for enhancing the binding efficiency or shortening the binding time thereof.

The particle size of the solid phase used in the present invention should preferably fall in the range from about 1 to about 100 μm . In addition, a suitable stirring operation according to the specific gravity of the particles is desirable for enhancing the contact probability between nucleic acids and the solid phase.

The isolation of the solid phase from a liquid in ~~the~~ step 4 according to the invention can be conducted by centrifugation or filtration. When the isolation step is automatized or mechanized, any of known B/F (bond form/free form) isolation techniques generally used in immunoassay devices utilizing antigen-antibody reactions can be employed.

~~The~~ ^S step 5 for washing the nucleic acid-binding solid phase with a solution containing a salt can be carried out by mixing the solid phase after completion of ~~the~~ step 4 with a salt-containing solution while maintaining the binding of nucleic acids to the solid phase, and isolating the solid phase from a liquid in a similar manner as ~~the~~ step 4.

~~The~~ ^S step 5 can be divided into ~~the~~ step 5a for removing non-nucleic-acid components being non-specifically bound to

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the solid phase from the solid phase, and ~~the~~ step 5b for removing the accelerator substance from the solid phase by washing. The liquid used in ~~the~~ step 5a or step 5b for washing the solid phase should be one which does not elute the binding nucleic acids from the solid phase, ^{as} ~~and~~ such property of the liquid affects the final yield of nucleic acids.

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Whereas ethanol solution having a concentration of 75% or more is not employed as the washing solution in the invention, GuHCl is preferably used as the solution in ~~the~~ step 5a as in ~~the~~ step 4. GuHCl has a denaturing activity, and the use of this substance in a concentration ranging from 4 to 6 mol/liter can remove non-specifically absorbed substances from the solid phase.

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As the washing solution used in ~~the~~ step 5b, those containing a salt which does not adversely affect the eluted nucleic acids and is capable of preparing an aqueous solution at a midrange concentration are desirable. This utilizes the nature that non-dissolved nucleic acids on the surface of the solid phase are insoluble in an aqueous solution containing a high concentration of a salt. By way of illustration, an aqueous solution of KOAc (potassium acetate) at a concentration of 500 mM or more is preferably employed. A mixture of an aqueous solution containing a salt and an alcohol can also be used in ~~the~~ step 5b.

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In the above case, the concentration of salt in the mixture can be reduced as compared with a single use of the salt, and such a mixture is permissive about the variation

of alcohol concentration due to volatilization of the alcohol component. The use of a 50% ethanol aqueous solution containing 50 mmol/liter or more of NaCl is advantageous in the invention.

When an alcohol-containing solution is used in ~~the~~ step 5b, addition of ~~a~~ step 7 after ~~the~~ step 6 is required for removing a trace alcohol component which may possibly contaminate the solution of purified nucleic acids. ~~The~~ ^Sstep 7 can be conducted by heating the solution, for instance.

~~The~~ ^Sstep 6 for eluting nucleic acids from the solid phase can be carried out by mixing the solid phase after washing with an equivalent volume or more of aqueous salt solution of a low concentration, or water. The nucleic acids migrate from the solid phase to an aqueous phase by this operation, and an aqueous solution of purified nucleic acids can be obtained by isolating the solid phase from the liquid in a similar manner to ~~the~~ step 4.

The aqueous salt solution of a low concentration or water used in the invention should preferably be sterilized, or be treated with DEPC (diethylpyrocarbonate) as necessary.

The aqueous salt solution of a low concentration or water in this step should preferably be used at a temperature of 55 to 60°C for enhancing the yield of nucleic acids, or similar advantages can be obtained when the container used for elution is heated at a similar temperature to the above.

For enhancing the yield of nucleic acids, ~~the~~ step 6 is

preferably conducted at least twice.

The washing procedure in ~~the~~ step 5 is carried out using a washing solution containing an acetate or chloride. Examples of the washing solution include an aqueous solution containing 0.5 mol/liter or more of potassium acetate and an aqueous solution containing 0.2 mol/liter of potassium chloride.

The washing solution used in ~~the~~ step 5b can be a solution containing 40% of ethanol and 10 mmol/liter or more of potassium acetate, or a solution containing 40% of ethanol and 25 mmol/liter or more of sodium chloride.

The solid phase bondable to nucleic acids used in ~~the~~ step 3 may be substances containing silicon dioxide.

The present invention as constructed above has the following advantages.

The invention can provide methods and apparatus for the recovery of nucleic acids, by which nucleic acids having a high purity can be rapidly and easily recovered from a material containing nucleic acids without deteriorating the yield, as compared with processes using washing steps with ^a single use of sodium chloride solution.

In other words, nucleic acids at a suitable purity for genetic tests or gene analyses can be rapidly and easily recovered without the use of hazardous substances.

In addition, methods and apparatus for the recovery of nucleic acids which are suitable for apparatus for the automatic recovery of nucleic acids from a biological sample can be provided.

B Further, methods and apparatus for the recovery of nucleic acids, which are suitable for apparatus for automatically recovering plasmid DNAs from *E. coli*, can be obtained.

B Other embodiments and variations will be obvious to those skilled in this art; this invention is not to be limited except as set forth in the following claims.